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Cramer, Mirjam ; Nagy, Ivana ; Murphy, Brian J ; Gassmann, Max ; Hottiger, Michael O ; Georgiev, Oleg ; Schaffner, Walter

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# NF- $\kappa$ B contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells

Mirjam Cramer<sup>1,a</sup>, Ivana Nagy<sup>1,a</sup>, Brian J. Murphy<sup>2</sup>, Max Gassmann<sup>3</sup>, Michael O. Hottiger<sup>4</sup>, Oleg Georgiev<sup>1</sup> and Walter Schaffner<sup>1,\*</sup>

<sup>1</sup>Institut für Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

<sup>2</sup>Biosciences Division, SRI International, Menlo Park, CA 94025, USA

<sup>3</sup>Institute for Veterinary Physiology, Vetsuisse Faculty and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland

<sup>4</sup>Institut für Veterinärbiochemie und Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

\*Corresponding author  
e-mail: walter.schaffner@molbio.unizh.ch

## Abstract

Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor family of cytokines that control vascular and lymphatic endothelium development. It has been implicated in promoting angiogenesis in pathological conditions via signaling to vascular endothelial growth factor receptor-1. PlGF expression is induced by hypoxia and proinflammatory stimuli. Metal responsive transcription factor 1 (MTF-1) was shown to take part in the hypoxic induction of PlGF in Ras-transformed mouse embryonic fibroblasts. Here we report that PlGF expression is also controlled by NF- $\kappa$ B. We identified several putative binding sites for NF- $\kappa$ B in the PlGF promoter/enhancer region by sequence analyses, and show binding and transcriptional activity of NF- $\kappa$ B p65 at these sites. Expression of NF- $\kappa$ B p65 from a plasmid vector in HEK293 cells caused a substantial increase of PlGF transcript levels. Furthermore, we found that hypoxic conditions induce nuclear translocation and interaction of MTF-1 and NF- $\kappa$ B p65 proteins, suggesting a role for this complex in hypoxia-induced transcription of PlGF.

**Keywords:** angiogenesis; hypoxia; inflammation; MTF-1; nuclear factor  $\kappa$ B; VEGF.

## Introduction

The placenta growth factor (PlGF) is a member of a family of structurally related angiogenic factors termed vascular

endothelial growth factors (VEGF). VEGF, also referred to as VEGF-A, is the founding member of this family and was identified due to its ability to promote vascular permeability as well as vascular endothelial cell proliferation and migration (Keck et al., 1989; Leung et al., 1989; Lyden et al., 2001; Nagy et al., 2002). Other family members include VEGF-B, VEGF-C, VEGF-D, VEGF-E and the endocrine gland-derived VEGF (EG-VEGF) (Yancopoulos et al., 2000; LeCouter et al., 2001). All factors are active as dimeric glycosylated proteins. Homo- and heterodimer formation involves cysteine residues that create a so-called 'cysteine knot' (McDonald and Hendrickson, 1993). VEGF binds to the tyrosine kinase receptors VEGFR-1 and VEGFR-2, and loss of VEGF-A or its receptors results in abnormal angiogenesis and lethality during development (Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999a,b). In contrast, PlGF binds only to VEGFR-1 (Park et al., 1994) and appears to be dispensable for normal development but crucial for angiogenesis during pathological conditions (Carmeliet et al., 2001; Luttun et al., 2002a; Odorisio et al., 2002). In humans, four isoforms of PlGF (PlGF-1 to 4) have been identified. They are generated by alternative splicing and differ in size and binding properties (Maglione et al., 1993; Cao et al., 1997; Yang et al., 2003).

PlGF gene expression is inducible upon proinflammatory stimuli as well as by the condition of low intracellular oxygen tensions, termed hypoxia (Green et al., 2001; Oura et al., 2003; Selvaraj et al., 2003). Several years ago, it was found that the transcriptional regulation of PlGF involves metal responsive transcription factor 1 (MTF-1, also termed metal response element binding transcription factor 1, or in short, metal transcription factor 1) as the hypoxic upregulation of PlGF mRNA was shown to be severely reduced in MTF-1<sup>-/-</sup> embryonic fibroblasts (Green et al., 2001). MTF-1, a protein that is conserved from insects to mammals, responds to diverse stress signals by translocating from the cytoplasm to the nucleus and binding to DNA sequence motifs termed metal response elements (MREs) (Brugnera et al., 1994; Lichtlen and Schaffner, 2001; Saydam et al., 2001; Zhang et al., 2001). The study revealed several putative MREs in the mouse and human PlGF upstream promoter region (Green et al., 2001). Also, the oxygen-dependent subunit of HIF-1, HIF-1 $\alpha$ , was proposed to play a role in the hypoxia-induced transcription of PlGF, based on experiments with a constitutively active form of HIF-1 $\alpha$  as well as HIF-1 $\alpha$ -deficient embryonic stem cells (Kelly et al., 2003). Zhang et al. (2003) reported on PlGF as a target gene of the forkhead/winged helix transcription factor FoxD1 (BF-2) in the developing kidney stroma and identified a conserved HNF3 $\beta$  binding site, which bound to bacterially-produced BF-2 protein in EMSA experiments.

<sup>a</sup>These authors contributed equally to this work.

Based on the proangiogenic role of PIGF in diverse (patho-)physiological conditions, we reasoned that additional factors are likely to be involved in its transcription. Angiogenesis occurs in physiological and pathological conditions and is tightly regulated. Inflammatory cells such as monocytes/macrophages or T lymphocytes are involved in this process by secreting cytokines that exert effects on endothelial cells (Naldini and Carraro, 2005). NF- $\kappa$ B is a key player in the inflammatory response and there are numerous reports on the role of NF- $\kappa$ B in angiogenic processes (Scatena et al., 1998; Malyankar et al., 2000; Klein et al., 2002; Ko et al., 2002; Patel et al., 2005).

In the present study, we report on the influence of NF- $\kappa$ B on the expression of PIGF in human embryonic kidney (HEK) 293 cells. We show that the PIGF promoter/enhancer region harbors DNA binding sites for NF- $\kappa$ B, which are bound by NF- $\kappa$ B in a transcriptionally active form. PIGF transcript levels rose substantially when NF- $\kappa$ B p65 was overexpressed in HEK293 cells and coexpression of NF- $\kappa$ B p65 and MTF-1 led to an even further increase. We also report on a nuclear translocation and interaction of NF- $\kappa$ B p65 and MTF-1 during hypoxic exposure and discuss a possible role of this complex in hypoxia-induced transcription of PIGF.

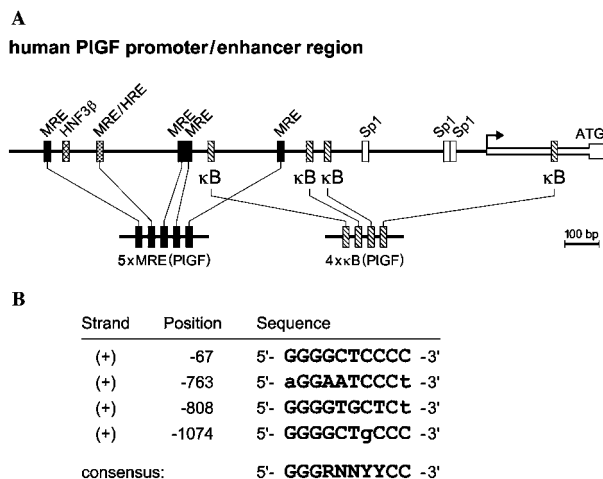
## Results

### Characterization of the PIGF promoter/enhancer region

A DNA segment of 1.7 kb length encompassing the 5'-flanking region of the human PIGF gene (GenBank accession number AC006530) was analyzed. Previously, multiple MREs had been identified in the PIGF promoter region (Green et al., 2001). A closer inspection of the sequence also revealed the presence of several potential  $\kappa$ B-sites (Figure 1).

### NF- $\kappa$ B associates with hPIGF promoter elements in a transcriptionally active form

To investigate binding of NF- $\kappa$ B to the putative  $\kappa$ B-elements, EMSA was performed using a probe combining all potential  $\kappa$ B-sites of the human PIGF gene (Figure 1). Figure 2A shows specific binding of NF- $\kappa$ B to this probe [4 $\times$  $\kappa$ B (PIGF)]. To analyze whether NF- $\kappa$ B can drive transcription from these sites, the combined  $\kappa$ B-elements, that were used for EMSA, were cloned into the OVEC (oligonucleotide vector)-reporter plasmid (Westin et al., 1987) and transcript levels were determined by nuclease S1 protection assays. The background reporter signal was somewhat increased when the empty reporter plasmid (OVEC-only) was co-transfected with pC-NF- $\kappa$ B p65 (Figure 2B, lane 2). This might reflect binding of NF- $\kappa$ B to some fortuitous  $\kappa$ B-site(s) in the backbone of the plasmid. The OVEC reporter gene system is generally accurate and reliable and was therefore used here as well, despite the elevated signal of the negative control, which had to be subtracted. When 4 $\times$  $\kappa$ B(PIGF)-OVEC was co-transfected with pC-NF- $\kappa$ B p65, the signal intensity markedly increased compared to OVEC-only (Figure 2B,



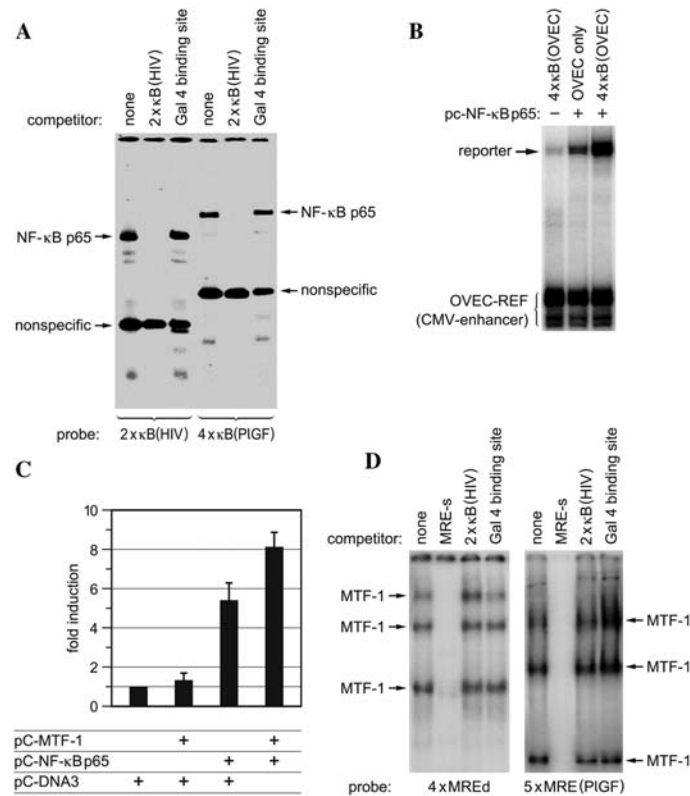
**Figure 1** Transcription factor binding elements in the human PIGF promoter/enhancer region.

(A) Schematic representation of the proximal promoter/enhancer region of the human PIGF gene with transcription factor binding sites indicated.  $\kappa$ B (NF- $\kappa$ B binding site, present study), Sp1 (Sp1 binding site, predicted in Green et al., 2001), MRE (metal-responsive element; Green et al., 2001, and present study), HRE (hypoxia-responsive element, predicted in Green et al., 2001), HNF3 $\beta$  (BF-2 binding site; Zhang et al., 2003). The four putative  $\kappa$ B sites and the five MREs, including three nucleotides of the respective flanking regions on each side, were combined to create the 4 $\times$  $\kappa$ B(PIGF) and 5 $\times$ MRE(PIGF) oligonucleotides, respectively, which were used in the experiments shown in Figure 2 (see also materials and methods). (B) Sequences of the putative  $\kappa$ B-sites in the human PIGF promoter/enhancer region. Core recognition sequences are indicated in bold; deviations from a commonly cited consensus (Leung et al., 2004) are indicated by lower case letters.

lane 3), which indicates binding and transcriptional activity of NF- $\kappa$ B from this 'mini-promoter'.

To test the responsiveness of the endogenous PIGF gene to NF- $\kappa$ B, quantitative RT-PCR was performed. PIGF transcript levels increased about fivefold upon transient expression of NF- $\kappa$ B p65 in HEK293 cells that were grown in 5% FBS (Figure 2C). MTF-1 was shown in earlier studies to activate PIGF transcription during hypoxia in Ras-transformed mouse embryonic fibroblasts (Green et al., 2001). However, exposure to 1% oxygen for 16 h did not show an effect on PIGF transcript levels in untransfected HEK293 cells or HEK293 cells overexpressing MTF-1 or NF- $\kappa$ B p65 alone or coexpressed (data not shown). The human placental cell line 3A-subE, human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were tested under the same hypoxic conditions but neither of these cell types showed an upregulation of PIGF transcripts by quantitative RT-PCR (data not shown). This probably reflects cell type specificity of the hypoxic induction of PIGF transcription.

Transient expression of MTF-1 did not lead to an upregulation of PIGF transcript levels in HEK293 cells (Figure 2C). Nevertheless, a further increase of PIGF transcript levels was observed when NF- $\kappa$ B p65 was co-expressed with MTF-1 (Figure 2C). To assess whether MTF-1 exerts its effect via binding to its designated DNA recognition sequences within the PIGF promoter/enhancer region, or rather exerts its effect via NF- $\kappa$ B and without DNA binding, EMSA was performed with a probe combining all



**Figure 2** NF- $\kappa$ B p65 binds to the PIGF promoter in a transcriptionally active form.

(A) Electrophoretic mobility shift assay (EMSA) showing DNA binding of NF- $\kappa$ B p65 to a probe that combines all potential  $\kappa$ B-sites of the human PIGF promoter/enhancer region [4 $\times$  $\kappa$ B (PIGF)] and to a probe combining the  $\kappa$ B-sites of HIV [2 $\times$  $\kappa$ B(HIV), positive control]. Specific binding was determined by competition with the unlabeled 2 $\times$  $\kappa$ B(HIV) oligonucleotide and an unspecific, unlabeled competitor (binding site for yeast Gal4). (B) S1 nuclease protection assay with the 4 $\times$  $\kappa$ B(PIGF)-OVEC reporter, the OVEC-only control and CMV-OVEC as reference. Cells were kept in 5% FBS prior to RNA isolation. The reporter signal ratio increased above the OVEC-only background upon overexpression of NF- $\kappa$ B p65. (C) Increased levels of endogenous PIGF transcript upon transient expression of NF- $\kappa$ B p65 and MTF-1 in HEK293 cells. Cells were transfected with 4  $\mu$ g of pC-NF- $\kappa$ B p65, pC-hMTF-1 or the empty vector (pC-DNA3). pC-DNA3 had been used for the cloning of both pC-NF- $\kappa$ B p65 and pC-hMTF-1. The final amount of transfected DNA was the same in all cases. Cells were kept in 5% FBS prior to RNA isolation. Transcript levels were determined by quantitative real-time PCR. TATA-binding protein (TBP), eukaryotic elongation factor 1A1 (EEF1A1) and transferrin receptor (TFRC) served as reference genes. The bars represent the mean of three independent experiments. (D) EMSA showing DNA binding of MTF-1 to a probe that combines all potential metal response elements of the human PIGF promoter/enhancer region [5 $\times$ MRE (PIGF)] and to a probe with four tandem repeats of MREd, a strong MRE of the mouse metallothionein I promoter. Several bands were detected in both cases, probably because some of the binding sites are preferentially bound by MTF-1. Specific binding was determined by competition with an unlabeled MRE-s oligonucleotide, and with unspecific, unlabeled competitors 2 $\times$  $\kappa$ B(HIV) and a binding site for yeast Gal4.

putative MREs [5 $\times$ MRE(PIGF), Figure 1]. Figure 2D shows that MTF-1 can bind to several of the MREs, suggesting that MTF-1 associates with the PIGF promoter by direct DNA binding.

Taken together, the results suggest a joint action of MTF-1/NF- $\kappa$ B p65 on the PIGF promoter and prompted us to test for protein interaction of the two factors.

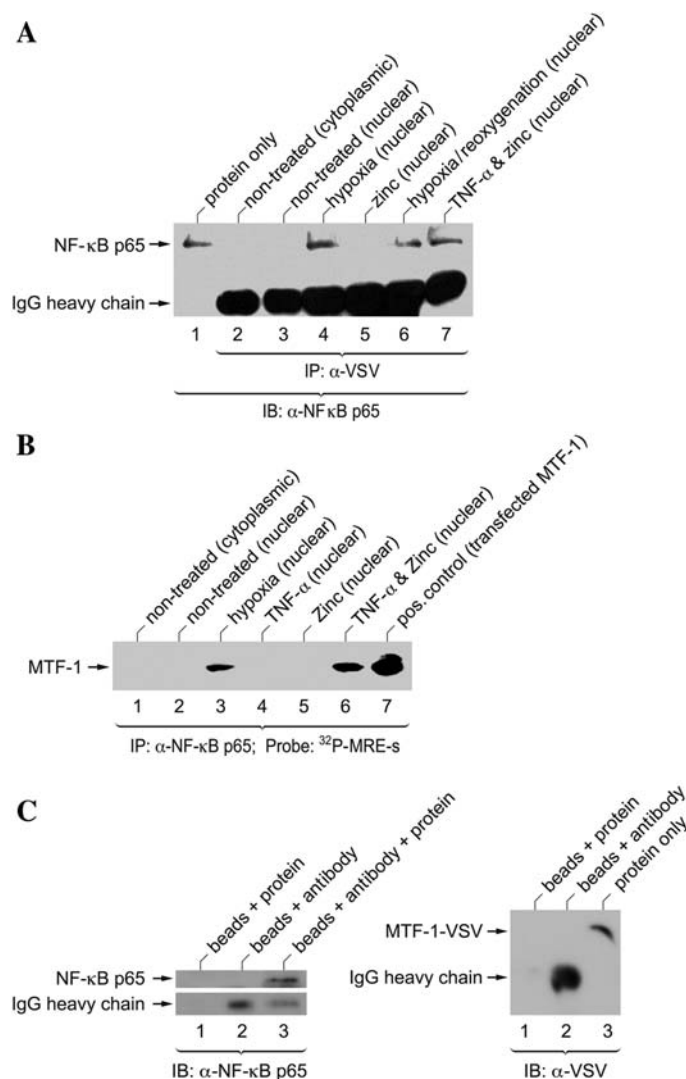
#### MTF-1 and NF- $\kappa$ B p65 interact in the nuclei of stimulated cells

To investigate a possible protein interaction between MTF-1 and NF- $\kappa$ B, co-immunoprecipitations and Southwestern analyses with nuclear and cytoplasmic HEK293 cell lysates were performed. In Southwestern analysis, co-immunoprecipitated endogenous MTF-1 was detected by binding to a labeled MRE oligonucleotide. Neither for overexpressed (Figure 3A) nor for endogenous proteins (Figure 3B) was an interaction observed in serum-starved, resting cells. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and zinc are known to be specific inducers of nuclear

translocation and activation of NF- $\kappa$ B and MTF-1, respectively. Simultaneous treatment of HEK293 cells with these inducers led to a detectable association of both overexpressed (Figure 3A) and endogenous (Figure 3B) MTF-1 and NF- $\kappa$ B p65 proteins in the nuclear fractions. The control experiments presented in Figure 3C ruled out nonspecific binding and nonspecific effects of reaction components. Single treatments with TNF- $\alpha$  or zinc did not promote the co-immunoprecipitation of MTF-1 or NF- $\kappa$ B p65, respectively (Figure 3A and B). Interestingly, the interaction between MTF-1 and NF- $\kappa$ B also occurred after hypoxic exposure of the cells (Figure 3A and B). Therefore, hypoxia seems to be a common stimulus for the nuclear translocation and interaction of MTF-1 and NF- $\kappa$ B.

#### Hypoxia induces nuclear localization of MTF-1 and NF- $\kappa$ B p65

To verify the effect of hypoxia on the cellular distribution of NF- $\kappa$ B p65 and MTF-1, immunofluorescence experi-



**Figure 3** NF- $\kappa$ B interacts with MTF-1 in the nuclei of stimulated cells.

(A) HEK293 cells were transfected with 5  $\mu$ g of pC-NF- $\kappa$ B p65 and VSV- (vesicular stomatitis virus) epitope-tagged pC-hMTF-1-VSV and serum-starved 24 h prior to various treatments of cells, as indicated. Immunoprecipitations (IP) were done with an anti-VSV antibody for MTF-1 and anti-NF- $\kappa$ B p65.  $\alpha$ , antibody; IB, immunoblot with indicated antibody. (B) Endogenous protein interaction was analyzed by Southwestern. HEK293 cells were serum-starved 24 h prior to preparations of nuclear and cytoplasmic lysates. NF- $\kappa$ B p65 was immunoprecipitated following treatments as indicated. MTF-1 was detected by hybridization to a radioactively labeled consensus MRE probe (designated MRE-s). (C) Controls with protein extracts of untransfected (left panel) and pC-MTF-1-VSV transfected (right panel) HEK293 cells, demonstrating specificity of the results shown in (A) and (B). Slot 1 reveals that neither NF- $\kappa$ B p65 nor MTF-1-VSV bind nonspecifically to protein A Sepharose beads. Slot 2 represents controls with beads and antibody (left panel:  $\alpha$ -NF- $\kappa$ B p65; right panel:  $\alpha$ -VSV), and without protein. Slot 3 shows that the protein extracts contained NF- $\kappa$ B p65 and MTF-1-VSV protein, respectively. NF- $\kappa$ B was enriched by immunoprecipitation for better detection (beads+antibody+protein).

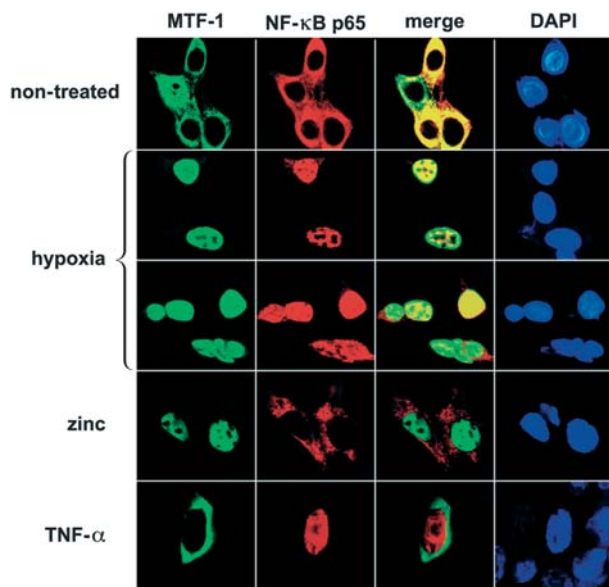
ments were performed. HEK293 cells were transiently transfected with expression vectors encoding NF- $\kappa$ B p65 and VSV-tagged MTF-1. Sixteen hours after transfection, cells were serum-starved in DMEM-BSA (0.5%) for 24 h. In untreated cells, both proteins localized predominantly to the cytoplasm (Figure 4). Elevated zinc concentrations caused nuclear translocation of MTF-1, but not of NF- $\kappa$ B p65. Conversely, TNF- $\alpha$  treatment induced rapid NF- $\kappa$ B p65 nuclear translocation, but had no effect on MTF-1, which remained cytoplasmic (Figure 4). However, when cells were exposed to hypoxia, nuclear translocation of both MTF-1 and NF- $\kappa$ B p65 was observed (Figure 4). These results show that in serum-deprived, normoxic HEK293 cells, NF- $\kappa$ B p65 and MTF-1 reside in the cyto-

plasm, while hypoxic conditions lead to nuclear translocation of both proteins.

## Discussion

The expression of placenta growth factor (PIGF) can potentially be controlled at several levels. A rather poorly understood step is the transcriptional regulation of the PIGF gene. Here we report that NF- $\kappa$ B is likely to be involved in this transcriptional regulation. NF- $\kappa$ B bound to  $\kappa$ B-sites of the PIGF gene *in vitro* and could drive transcription of PIGF when transiently expressed in HEK293 cells. We found that coexpressing NF- $\kappa$ B p65 and MTF-





**Figure 4** Hypoxia induces nuclear translocation of MTF-1 and NF- $\kappa$ B p65.

Immunofluorescence experiments showing subcellular distribution of NF- $\kappa$ B p65 and MTF-1. pC-hMTF-VSV and, to obtain similar fluorescence signal intensities, also pC-NF- $\kappa$ B p65 were transfected into HEK293 cells. Cells were serum-starved for 24 h and either left untreated, exposed to hypoxia (4 h), or treated with 200  $\mu$ M ZnCl<sub>2</sub> (2 h) or 20 ng/ml TNF- $\alpha$  (30 min). MTF-1 is shown in green (left panel), NF- $\kappa$ B p65 is shown in red (second left panel). DNA was stained with 4',6'-diamino-2-phenylindole (DAPI, right panel, blue).

1, a protein with an established role in PIGF transcription, resulted in a further increase of PIGF transcript levels. The two proteins show a nuclear interaction and hypoxia is a common stimulus for nuclear translocation and interaction of NF- $\kappa$ B and MTF-1.

Evidence has accumulated for a role of NF- $\kappa$ B in angiogenic processes during hypoxia and inflammation (Klein et al., 2002; Ko et al., 2002; Kofler et al., 2005; Patel et al., 2005). Our findings suggest that NF- $\kappa$ B signaling can promote its proangiogenic effects by upregulating PIGF transcription. It was previously demonstrated that NF- $\kappa$ B regulates proangiogenic molecules such as IL-8 and the best-studied VEGF family member, VEGF-A (Mukaida et al., 1994; Huang et al., 2000; Ko et al., 2002). Dominant negative forms of NF- $\kappa$ B/RelA decreased VEGF mRNA levels in human ovarian and breast cancer cells (Huang et al., 2000; Shibata et al., 2002). Advanced glycation end products (AGE), which form at an accelerated rate in diabetes, induce angiogenesis by signaling to VEGF via NF- $\kappa$ B (Okamoto et al., 2002). It was reported that PIGF potentiates bioactivity of VEGF (Park et al., 1994), possibly by the formation of heterodimers. A common regulatory signaling pathway could provide a mechanism to coordinate the interplay of the two factors.

Our studies hint at an involvement of an NF- $\kappa$ B/MTF-1 complex in the transcription of PIGF under hypoxic conditions, while a role of MTF-1 in the transcription of PIGF was reported before. The previous MTF-1 study was undertaken in Ras-transformed MEFs and demonstrated the necessity of MTF-1 for the hypoxic upregulation of

PIGF in this cell type. Recently, it was shown that MTF-1 null xenograft tumors had decreased tumor vessel densities as compared to wild-type xenografts, which might reflect the positive contribution of MTF-1 to PIGF expression (Haroon et al., 2004). We detected a basal level of PIGF transcripts in HEK293 cells, which was stimulated by both overexpression of NF- $\kappa$ B p65 and coexpression of NF- $\kappa$ B p65 and MTF-1 (Figure 2C). Yet, in either case we could not observe a hypoxic induction of PIGF transcription (data not shown), demonstrating cell type specificity of this process. Nevertheless, we observed nuclear translocation and interaction of NF- $\kappa$ B and MTF-1 upon hypoxia in HEK293 cells. Regarding the additive effect of NF- $\kappa$ B and MTF-1 coexpression on PIGF transcript levels, the results suggest a joint action of the two factors on the PIGF promoter, which might play a role in the hypoxic induction of PIGF in certain cell types. However, if both factors would be involved in the hypoxic upregulation of PIGF, the formation of an NF- $\kappa$ B/MTF-1 protein complex would not constitute by itself the specificity of the hypoxic signal. This is also underlined by the observation that this interaction was not only taking place in HEK293 cells subjected to hypoxia, but also in cells that had been treated simultaneously with TNF- $\alpha$  and zinc (Figure 3). Interestingly, Kelly et al. (2003) have implicated a role of HIF-1 $\alpha$  for the hypoxic upregulation of PIGF. In light of the data presented here, it appears likely that different factors help to activate the PIGF promoter/enhancer region under hypoxia, possibly in a cell-type-specific manner. In line with such a cell-type specificity, hypoxic upregulation of VEGF transcription, commonly referred to as a HIF-dependent process, was shown to be HIF-1-independent in colon cancer cells (Mizukami et al., 2004). Moreover, hypoxia-induced but HIF-independent upregulation of erythroid 5-aminolevulinic synthase (Hofer et al., 2003) suggests the existence of different mechanisms for this process in given cell types.

Expression of PIGF and other VEGFs is certainly a complex process with several levels of regulation and it has to be assessed for each of the diverse (patho-)physiological conditions where VEGFs play a role. The results presented here constitute a basic finding and could, along with further studies, give new insights into the regulation of PIGF expression during inflammation and hypoxia. PIGF expression has been proposed as a therapeutic target, on one hand to induce revascularization of ischemic tissues, and on the other hand to block uncontrolled angiogenesis and inflammation in cancer and other diseases (Luttun et al., 2002b,c).

## Materials and methods

### Cell cultures and treatments

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, USA). For serum-starvation, FBS was replaced by 0.5% bovine serum albumin (BSA). Transfections were done by the calcium-phosphate method on 100-mm-diameter dishes at 60% cell confluency. Where indicated, cells were subjected to hypoxia

(1% O<sub>2</sub>), treated with 200  $\mu$ M ZnCl<sub>2</sub> and/or human TNF- $\alpha$  (20 ng/ml; R&D Systems, Minneapolis, USA) for up to 16 h.

### Electrophoretic gel mobility shift assay (EMSA)

EMSAs were performed as described previously (Gunes et al., 1998). Nuclear protein extracts of HEK293 cells, transfected with 10  $\mu$ g of pC-NF- $\kappa$ B p65 or pC-hMTF-1, were used for binding reactions. To generate 4 $\times$  $\kappa$ B(PIGF), the following oligomers were first cloned into SacI/XhoI digested pGL3-basic (Promega, Madison, USA) and subsequently cut out by appropriate restriction enzymes: 5'-CGA GCC ACA GGG GGC TGC CCC GCA GGC AGG GGT GCT CTC GGA GGC-3', 5'-ACC CCT GCC TGC GGG GCA GCC CCC TGT GGC TCG AGC T-3', 5'-TAG GAA TCC CTT GGC GCC AGG GGC TCC CCG GGG GAG-3' and 5'-TCG ACT CCC CCG GGG AGC CCC TGG CGC CAA GGG ATT CCT AGC CTC CGA GAG C-3'. To generate 5 $\times$ MRE(PIGF) the same strategy was employed using the following oligomers: 5'-CGA GGT TCC TGC AGC CTG GGG CAG GCG TGC AGA CTC ACA TGC CCA TGC ACA CG-3', 5'-ATG TGA GTC TGC ACG CCT GCC CCA GGC TGC AGG AAC CTC GAG CT-3', 5'-CGC AGA TGC ACA CAG ACC CAC AGA TGC GCG CAC ACA CAC G-3' and 5'-TCG ACG TGT GTG TGC GCG CAT CTG TGG GTC TGT GTG CAT CTG CGC GTG TGC ATG GGC-3'. Sequences of control probes (sense strands): 2 $\times$  $\kappa$ B(HIV): 5'-AAT TCG GGA CTT TCC CAT CGG GAC TTT CC-3'; MRE-s: 5'-CGA GGG AGC TCT GCA CAC GGC CCG AAA AGT G-3'; yeast Gal4 binding site: 5'-TCC GGA GGA CTG TCC TCC GG-3'.

### S1 nuclease protection assay

The S1 nuclease protection assay was performed as described previously (Weaver and Weissmann, 1979; Westin et al., 1987). To generate the reporter gene 4 $\times$  $\kappa$ B(PIGF)-OVEC, the same oligomers as described for EMSA were cloned into SacI/Sal I-digested pOVEC (Westin et al., 1987). HEK293 cells were transfected with 10  $\mu$ g reporter plasmid, 0.5  $\mu$ g reference plasmid (CMV-OVEC-REF) and 4  $\mu$ g of pC-NF- $\kappa$ B p65 or pC-DNA3. The gels were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

### Quantitative real-time PCR

HEK293 cells were transfected with 4  $\mu$ g of pC-NF- $\kappa$ B p65, pC-hMTF-1 or pC-DNA3. Precipitates were washed off after 14 h and cells harvested after an additional 24 h incubation in 5% FBS. After isolation (RNeasy Mini Kit, Qiagen, Hilden, Germany), 10  $\mu$ g RNA were treated with DNase (DNA-free™, Ambion, Austin, USA) according to the manufacturer's protocol. Purity and integrity of RNA was assessed on the Bioanalyzer (Agilent, Palo Alto, USA). Four-hundred ng RNA were used for cDNA synthesis by Superscript™ II RNase H Reverse Transcriptase (Invitrogen) following the supplier's instructions. Quantitative real-time PCRs were performed on the ABI PRISM 7900HT Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) with primers as determined by Primer Express® software. PIGF primers were designed to potentially amplify transcripts of all known isoforms. PIGF: 5'-TGT TCA GCC CAT CCT GTG TC-3' and 5'-ACA GTG CAG ATT CTC ATC GCC-3'. Reference genes: TFRC (transferrin receptor): 5'-CAT TTG TGA GGG ATC TGA ACC A-3' and 5'-CGA GCA GAA TAC AGC CAC TGT AA-3'; TBP (TATA-binding protein): 5'-GCC CGA AAC GCC GAA TAT A-3' and 5'-CGT GGC TCT CTT ATC CTC ATG A-3'; EEF1A1 (eukaryotic elongation factor 1A1): 5'-AGC AAA AAT GAC CCA CCA ATG-3' and 5'-GGC CTG GAT GGT TCA GGA TA-3'. PCR efficiency was determined by the means of standard curves and data analyzed with SDS Software v2.2

by the  $\Delta\Delta$ Ct method and taking the geometric mean of the  $\Delta\Delta$ Ct values from the three reference genes. In given conditions, TFRC expression has been reported to respond to inflammatory processes (Tomkins, 2003) and might therefore be influenced by overexpression of NF- $\kappa$ B p65. However, the change of TFRC transcript levels in pC-DNA3 (empty control vector) transfected versus NF- $\kappa$ B p65 overexpressing HEK293 cells was as low as 1.12 $\pm$ 0.07-fold (average with standard deviation, measurements in triplicate,  $n=3$ ), most likely reflecting small experimental fluctuations and not a physiological effect. Analysis and calculations for TFRC were done as described above. EEF1A1 and TBP served as reference genes.

### Co-immunoprecipitation (Co-IP) and immunoblotting

The antibody against NF- $\kappa$ B p65 (sc-372) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The anti-VSV (vesicular stomatitis virus) epitope-tagged MTF-1 was purchased from Sigma (St. Louis, USA; V5507). Cell lysates were prepared in 20 mM HEPES pH 7.9, 25% glycerol, 0.25 M NaCl, 1 mM EDTA for nuclear fractions, and in 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA for cytoplasmic fractions, supplemented with 2.5 mM DTT, 5 mg/ml leupeptin and 1.2 mM PMSF. Four-hundred  $\mu$ g total protein were used for Co-IP and incubated with 2.5  $\mu$ l of antibody for 1 h, followed by 30 min incubation with 50% (v/v) Protein A sepharose. Precipitates were washed six times with EBC-170 and fractionated on 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, USA).

### Southwestern blot analysis

Southwestern blot analysis was done as previously described (Radtko et al., 1993), except that proteins were immunoprecipitated with  $\alpha$ -NF- $\kappa$ B p65 antibody prior to fractionation on SDS-polyacrylamide gels.

### Indirect immunofluorescence

Indirect immunofluorescence and fluorescence microscopy were performed as described previously (Saydam et al., 2001). The antibody for NF- $\kappa$ B p65 was purchased from Santa Cruz Biotechnology (sc-372).

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